J. Insect Physiol., 1974, *Vol.* 20, *pp.* 1943 *to* 1948. *Pergamon Press. Printed in Great Britain*

SPECTROPHOTOMETRIC MEASUREMENT OF JUVENILE HORMONE BINDING IN SUBCELLULAR COMPONENTS OF THE INDIAN MEAL MOTH*

S. M. FERKOVICH,† R. R. RUTTER,† and D. W. ANTHONY‡

Agriculture Research Service, U.S. Department of Agriculture

(*Received* 15 *March* 1974)

Abstract—The relative degree of juvenile hormone binding to various subcellular fractions of larvae of the Indian meal moth, *Plodia interpunctella*, was studied by u.v. difference spectroscopy. Difference absorbance changes at 280 nm due to binding of the hormone to protein(s) were obtained in six particulate fractions from a sucrose density gradient and in the supernatant and microsomal fractions. A low-density, particulate fraction primarily containing membrane fragments, electron dense bodies (200 nm), and RNA was most active in binding the hormone.

INTRODUCTION

THE SITE of juvenile hormone (JH) action at the cellular and subcellular levels is not known though evidence exists that the hormone interacts directly on the cell nucleus and thus effects regulation of transcription (SLAMA, 1971), or translation of genetic information (ILAN *et al.*, 1970). This interaction may also involve alterations in the permeability of cell and nuclear membranes (KROEGER, 1968; BAUMANN, 1969; WIGGLESWORTH, 1969). WILLIAMS and KAFATOS (1971) suggested that JH may also act indirectly by interaction with other subcellular components.

To elucidate the mechanism of action of JH, we need information concerning the initial interaction of the hormone with cellular and subcellular components of insect tissue and the characteristics of such an interaction. Also, as a regulator of growth and development, JH may have multiple sites of action for each of its major physiological effects as with certain steroids (TATA, 1970) or may have a single site of action leading to all the different events. For example, JH was recently reported to directly affect mitochondrial metabolism in the Indian meal moth, *Plodia interpunctella* (Hübner) (FIRSTENBERG and SILHACEK, 1973). Also, SCHMIALEK (1973) reported the binding of a radiolabelled analogue of JH by a ribonucleoprotein receptor in epidermal tissue of pupal *Tenebrio molitor* L., though the possible function of the receptor was not described. In addition, studies of the mechanism of action of steroid hormones by LISK (1971) demonstrated that the

*Mention of a proprietary product in this paper does not constitute an endorsement of the product by the USDA.

†Insect Attractants, Behavior and Basic Biology Research Laboratory, Gainesville, Florida 32604.

‡Insects Affecting Man Research Laboratory, Gainesville, Florida 32604.

initial biochemical responses of target tissue are effected by binding to specific receptors that have a high affinity for the hormones. Theoretically, therefore, the primary sites of action of JH could be determined by the relative binding affinity of subcellular fractions of insect tissue for the hormone; and difference spectroscopy has been effectively used to follow changes in molecular conformation caused by the binding of certain olfactory and gustatory stimulants by isolated, biologically active macromolecules (DASTOLI and PRICE, 1966; ASH and SKOGEN, 1970; NORRIS *et al.*, 1971; FERKOVICH *et al.*, 1973). We thus used the technique of difference spectroscopy to study the binding of JH by subcellular fractions of the Indian meal moth.

MATERIALS AND METHODS

Subcellular fractionation of Plodia *larvae*

Larvae were reared on a defined diet with a controlled temperature and r.h. as described by SILHACEK and MILLER (1972).

Eleven-day-old, fifth instar larvae (7 mg average wt./larva) were fractionated according to a centrifugation procedure patterned after NORRIS *et al.* (1971). Briefly, 0·5 g of fifth instar larvae were homogenized in 5 ml of 0·5 M sucrose in 0·05 M Tris–HCl, pH 7·5, and centrifuged at 20,000 ***g*** for 45 min. The supernatant (soluble proteins and microsomes) was decanted and saved. The pellet was resuspended in 5 ml of 0·8 M sucrose and layered on a discontinuous gradient composed of 2·5 ml each of 1·0, 1·2, 1·5, and 1·8 M sucrose buffered with Tris–HCl, pH 7·5. After centrifugation at 90,000 ***g*** (R max) for 2 hr, the fractions were collected, diluted to 50 ml with Tris-HCl buffer, and centrifuged at 90,000 ***g*** (R max) for 1 hr. The resultant pellets were each resuspended in 4 ml of buffer.

Nucleic acid and protein analyses

The protein content of each fraction was measured by the method of LOWRY *et al.* (1951).

Certain fractions were extracted according to ZAMENHOF *et al.* (1964), and the content of DNA and RNA was determined as described by CERIOTTI (1952, 1955).

Electron microscopy

For ultrastructural identification of the fractions, another set of pellets was prepared in the same manner as described. However, these pellets were fixed in 4·0% glutaraldehyde and then in 1·0% osmium tetraoxide (in phosphate buffer pH 7·2), dehydrated through graded acetone solution, and embedded in Eponaraldite (MOLLENHAUR, 1964). The sections were then stained with uranyl acetate and lead citrate (VENABLE and COGGESHALL, 1965) and examined in an electron microscope.

Spectrophotometric analysis

Binding of JH was measured spectrophotometrically as described by FERKOVICH *et al.* (1973). The difference absorbance induced at 280 nm upon addition of the hormone to each fraction of the homogenate was recorded by using a Gilford model 2000 single-beam recording photometer coupled to a Beckman D.U. monochromator. Baselines (zero absorbance) were attained by electronically negating

the absorbance of the fraction and the hormone with the absorbance control system (total absorbance negated was less than 2·0 Å units). Full-scale sensitivity of the recording system was set at 0 to 0·5 Å, and the temperature of the cell compartment was held by thermostat at 20°C. The difference absorption change was measured at 280 nm because the region 278 to 281 nm is characteristically the maximum peak area for proteins (HERSKOVITS, 1967).

Stock solutions of a mixture of isomers of *Cecropia* juvenile hormone (methyl 10,11-epoxy-ethyl-3,11-dimethyl-2,6-tridecadienoate) (Hoffmann–La Roche) were either prepared by dispersion in double-distilled water by sonication according to FERKOVICH *et al.* (1973) or by dissolving the JH in absolute ethanol so that 25μl of the sonicated solution or 5μl of the ethanolic solution, when mixed with Tris–HCl buffer, pH 7·5, resulted in a concentration of 5×10^{-4} M JH.

Characterization of reactant materials

To determine whether the absorbance changes at 280 nm were due to interaction with protein, we heated a preparation at 64°C for 10 to 30 min before addition of the hormone. In some cases, a preparation was preincubated with the sulphydryl group inhibitor, N–ethylmaleimide (NEM), in acetone or ethanol at 1×10^{-3} M for 20 min at 20°C.

RESULTS

Interaction of juvenile hormone with subcellular fractions

The change in absorbance induced by the JH in the predominant subcellular components identified in each fraction is shown in Table 1. A time-dependent positive change occurred when JH was added to aliquots of five of the fractions obtained from the discontinuous density gradient and to the supernatant (soluble proteins and microsomes). However, the greatest change occurred with fraction F_1; less occurred with fraction F_2; that with F_3 and F_4 was similar but less than that with

TABLE 1—CHANGE IN DIFFERENCE ABSORBANCE PRODUCED BY ADDING 5×10^{-4} M JH TO 20 μg protein/ml OF EACH FRACTION FROM THE WHOLE LARVAL HOMOGENATE

Fraction F	Subcellular components identified	+ Δ Absorbance at 280 nm/5 min
F_1	Membrane fragments and vesicles, electron dense bodies (200 nm dia.), few small mitochondria	0·104
F_2	Fragments of rough endoplasmic reticulum, few electron dense bodies (200 nm dia.), few small mitochondria	0·074
F_3	Fragments of rough endoplasmic reticulum, large mitochondria, few lysosomes	0·059
F_4	Nuclear material, large membrane fragments, few large mitochondria, possibly Golgi vesicles	0·053
F_5	Large tissue fragments (e.g. muscle and cuticle)	0·037
Supernatant	Soluble proteins and microsomes	0·028
Microsomes	Microsomes	0·050*

*Negative Δ A at 280 nm/5 min.

fractions F_1 and F_2; and the absorbance change with F_5 was less than that occurring in any other particulate fractions from the density gradient. The absorbance change associated with the supernatant was less than that obtained with any of the particulate fractions.

Although JH thus induced a change in absorbance in the supernatant, we did not know whether JH would produce an absorbance change (bind) in the microsomal fraction alone. To determine this, we centrifuged the supernatant at 105,000 *g* for 2 hr and added the JH to the resulting fraction. In this case, the JH caused a negative change in absorbance (Table 1).

Characterization of reactant materials

The next step was to determine whether the absorbance change at 280 nm involved an interaction of JH with proteins. We therefore investigated the effects of the sulphydryl group inhibitor, NEM, and heat on the activity of fractions F_1 and F_2 prepared as previously described. NEM reduced the magnitude of the change produced by JH to below that produced by acetone in both F_1 and F_2 (Table 2); heating destroyed the activity of both. The analyses for RNA, DNA,

TABLE 2—INHIBITION OF THE JH-INDUCED CHANGE IN ABSORBANCE IN FRACTIONS F_1 AND F_2 (20 μg protein) DUE TO HEAT TREATMENT OR PREINCUBATION OF THE FRACTION WITH NEM PRIOR TO ADDITION OF 5×10^{-4} M JH

+ Δ absorbance at 280 nm/5 min	Treatment
	F_1
0·095	
0·070	10 μl acetone
0·025	10 μl NEM (10^{-3} M)
0·000	Boiling water-bath (15 min)
	F_2
0·085	
0·068	10μl acetone
0·045	10 μl NEM (10^{-3} M)
0·000	Boiling water-bath (15 min)

and protein revealed that in fraction F_1, the ratios of μg protein/μg RNA and μg protein/μg DNA were 50 and 100, respectively. The corresponding values for fraction F_2 were 30 and 300.

Also, treatment of the microsomal fraction with a sulphydryl group inhibitor (10^{-3} M NEM) or with trypsin (10 μg/ml) at 25°C for 30 min or heating the fraction at 64°C for 30 min destroyed activity.

Finally, we were interested in knowing whether JH would induce an absorbance change in bovine serum albumin. Since bovine serum albumin was reported to bind and thus solubilize JH (FIRSTENBERG and SILHACEK, 1973; RIDDIFORD and AJAMI, 1973), any changes produced by JH would be due to JH binding to the BSA. We found that the addition of JH (5×10^{-4} M) to 100 and to 125 μg of

BSA/ml produced time-dependent positive absorbance changes of 0·010 and 0·019/10 min, respectively.

DISCUSSION

Ultraviolet difference spectroscopy has been effectively used to measure binding of olfactory and gustatory stimulants by functionally active macromolecules (DASTOLI and PRICE, 1966; ASH and SKOGEN, 1970; NORRIS *et al.*, 1971; FERKOVICH *et al.*, 1973). Although the technique of u.v. difference spectroscopy has been used to distinguish between enzymatic and non-enzymatic binding (ASH, 1968), we did not attempt to make this distinction.

We did detect JH binding in the microsomal and the soluble protein fractions and in all the particulate fractions from the sucrose density gradient; the greatest degree was associated with the fraction (F_1) that contained membrane-bound protein and RNA, possibly a ribonucleoprotein. Binding could be prevented by adding sulphydryl group inhibitors or by heating. SCHMIALEK (1973), too, reported that a radiolabelled analogue of JH selectively bound to a Triton X–100 solubilized ribonucleoprotein in epidermal tissue from *T. molitor*. Since a protein-modifying agent was effective in preventing binding in both cases, we suspect that our F_1 fraction contained a binding protein similar to that described by SCHMIALEK.

Evidence that the primary site of action of JH is the cell nucleus was reviewed by SLÁMA (1971). Our results indicated a relatively low degree of binding in the fraction containing nuclear material; however, no whole nuclei were evident (the fraction consisted of smeared nuclear material and large membrane fragments, likely resulting from disruption of nuclei during the fractionation procedure).

Finally, as one would expect, JH binding was detected in the supernatant (soluble proteins) and in the microsomal fraction. Proteins that bind and transport JH (TRAUTMANN, 1972; WHITMORE and GILBERT, 1972; EMMERICH and HARTMANN, 1973) and the enzymes (NEMEC, 1972; WHITMORE *et al.*, 1972) that deactivate the hormone in the haemolymph (soluble proteins) have been reported. TERRIERE and YU (1973) found that microsomal preparations from houseflies, *Musca domestica*, metabolized two JH analogues.

REFERENCES

ASH K. O. (1968) Chemical sensing: an approach to biological molecular mechanisms using difference spectroscopy. *Science, Wash.* **162**, 452–454.

ASH K. O. and SKOGEN J. D. (1970) Chemosensing; selectivity, sensitivity and additive effects on a stimulant-induced activity of olfactory preparations. *J. Neurochem.* **17**, 1143–1153.

BAUMANN G. (1969) Juvenile hormone: effect on biomolecular lipid membranes. *Nature, Lond.* **223**, 316–317.

CERIOTTI G. (1952) A microchemical determination of deoxyribonucleic acid. *J. biol. Chem.* **198**, 297–303.

CERIOTTI G. (1955) Determination of nucleic acids in animal tissues. *J. biol. Chem.* **214**, 59–70.

DASTOLI F. R. and PRICE S. (1966) Sweet-sensitive protein from bovine taste buds: isolation and assay. *Science, Wash.* **154**, 905–907.

EMMERICK H. and HARTMANN R. (1973) A carrier lipoprotein for juvenile hormone in the haemolymph of *Locusta migratoria*. *J. Insect Physiol.* **19**, 1663–1675.

FERKOVICH S. M., MAYER M. S., and RUTTER R. R. (1973) Sex pheromone of the cabbage looper: reactions with antennal proteins *in vitro*. *J. Insect Physiol.* **19**, 2231–2243.

FIRSTENBERG D. E. and SILHACEK D. L. (1973) Juvenile hormone regulation of oxidative metabolism in isolated insect mitochondria. *Experientia* **29**, 1420–1422.

HERSKOVITS T. T. (1967) Difference spectroscopy. In *Methods in Enzymology* (Ed. by HIRS C. H. W.) **11**, 748–755. Academic Press, New York.

ILAN J., ILAN J., and PATEL N. (1970) Juvenile hormone determination of translational control through transfer ribonucleic acid and enzyme. *J. biol. Chem.* **245**, 1275–1281.

KROEGER H. (1968) Gene activities during insect metamorphosis and their control by hormones. In *Metamorphosis; A Problem in Developmental Biology* (Ed. by ETKIN W. and GILBERT L. I.), pp. 185–219. Appleton–Century–Crofts, New York.

LISK R. D. (1971) The physiology of hormone receptors. *Am. Zool.* **11**, 755–767.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L., and RANDALL R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MOLLENHAUR H. (1964) Plastic embedding mixture for use in electron microscopy. *Stain Technol.* **39**, 111–114.

NEMEC V. (1972) The effects of juvenile hormone analogue on the esterase activity in the haemolymph of *Pyrrhocoris apterus* (L.) larvae ((Hemiptera). *Acta ent. Bohem.* **69**, 137–142.

NORRIS D. M., FERKOVICH S. M., BAKER J. E., ROZENTAL J. M., and BORG T. K. (1971) Energy transduction in quinone inhibition of insect feeding. *J. Insect Physiol.* **17**, 85–97.

RIDDIFORD L. M. and AJAMI A. M. (1973) Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol.* **19**, 749–762.

SCHMIALEK P. (1973) Ribonucleoprotein particle in epidermis cells as the receptor for juvenile hormone. *Nature, Lond.* **245**, 267–268.

SILHACEK D. L. and MILLER G. L. (1972) Growth and development of the Indian meal moth, *Plodia interpunctella* (Lepidoptera: Phycitidae) under laboratory mass-rearing conditions. *Ann. ent. Soc. Am.* **65**, 1084–1087.

SLÁMA K. (1971) Insect juvenile hormone analogs. *A. Rev. Biochem.* **40**, 1079–1102.

TATA J. R. (1970) Regulation of protein synthesis by growth and development hormones. In *Biochemical Action* of *Hormones* (Ed. by LITWACK G.), pp. 89–133. Academic Press, New York.

TERRIERE L. C. and YU S. J. (1973) Insect juvenile hormone: induction of detoxifying enzymes in the housefly and detoxication by housefly enzymes. *Pestic. Biochem. & Physiol.* **3**, 96–107.

TRAUTMANN K. H. (1972) *In vitro*-Studium der Trägerprotein von ^{3}H-markierten juvenilhormonwirksamen Verbindungen in der Hämolymphe von *Tenebrio molitor* L. Larven. *Z. Naturf.* **276**, 263–273.

VENABLE J. H. and COGGESHALL R. (1965) A simplified lead citrate stain for use in electron microscopy. *J. cell Biol.* **25**, 407–408.

WHITMORE E. and GILBERT L. I. (1972) Haemolymph lipoprotein transport of juvenile hormone. *J. Insect Physiol.* **18**, 1153–1167.

WHITMORE D., WHITMORE E., and GILBERT L. (1972) Juvenile hormone induction of esterases: a mechanism for the regulation of juvenile hormone titre. *Proc. nat. Acad. Sci. U.S.A.* **69**, 1592–1595.

WIGGLESWORTH V. B. (1969) Chemical structure and juvenile hormone activity: comparative tests on *Rhodnius prolixus*. *J. Insect Physiol.* **15**, 73–94.

WILLIAMS C. M. and KAFATOS F. C. (1971) Theoretical aspects of the action of juvenile hormone. *Bull. Soc. ent. suisse* **44**, 151–162.

ZAMENHOF S., BURSZTYN H., RICH K., and ZAMENHOF P. J. (1964) The determination of deoxyribonucleic acid and of cell number in brain. *J. Neurochem.* **11**, 505–509.